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FINAL REPORT

on

SPAR PROJECT: COUNTER CURRENT DISTRIBUTION OF BIOLOGICALS

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COUNTERCURRENT DISTRIBUTION
PRELIMINARY REPORT

SUMMARY

Preliminary ground-based experiments strongly support the validity of a proposed space experiment in the separation of biological cells by counter current distribution (CCD). It is shown that a low-level applied selective field (for example as 5 V/cm) can accelerate the separation of the phases by a very large factor (for example 35-fold). An electric field therefore should be very effective in space in lieu of gravity for the phase separation step. This appears to solve a central problem in implementing CCD in space and opens the technique to many types of large cell mixtures for which it has not been applicable before due to rapid settling effects. It is shown that electrophoretic migration of the cells, as a possibly disturbing factor, will be of negligible effect. The phase forming polymers used in the preliminary experiments are not, per se, compatible with biological cells. However, other suitable polymers are already under development, and are predicted to give similar rapid phase separation. The present experiments were implemented with a prototype chamber similar to that proposed for space. Attenuation of a light beam by scattering generated a signal indicating the course of the phase separation. The highly promising results of these experiments now appear clearly to justify a SPAR experiment as a next logical phase of investigation.

INTRODUCTION

Following the submission of our proposal "Counter Current Distribution of Biologicals" in response to A.O. No. OA-76-02 of the Space Processing Rocket Experiment Project, a contract (NAS8-32353) was awarded to the University of Oregon Health Sciences Center to allow further definition and development of the flight experiment. The Statement of Work in that contract included the following tasks:

1. Verify that there is an aqueous polymer system that is significantly separated by an applied electric field. Within the limits imposed by 1 g conditions, analyze field-induced phase separation and the influence the field and separating phases have on the biological cells of interest. Design ground experiments to assess experiment operation in space.
2. Do a detailed analysis of the equipment proposed for the SPAR to show that the proposed components can accomplish the planned objectives. In particular, analyze (a) the removal of bubbles, and (b) optical devices proposed or otherwise available to measure the progression of phase separation.
3. Supply general operating parameters for an electric field-driven CCD apparatus as determined by 1 g experiments.

The principal results obtained during this study are summarized below. They verify that the approach we have proposed is a valid and promising one and that a SPAR flight experiment is indeed an appropriate next phase of experimentation.

ELECTRIC FIELD-DRIVEN PHASE SEPARATION

The central problem to be solved if phase-separated aqueous polymer systems are to be used as cell partition media in space is that of finding a method for

separating the emulsified phases in the effective absence of gravity-driven settling. We proposed to use an applied electric field as the driving force since droplets of one phase suspended in the other are known to exhibit electrophoretic mobilities which increased linearly with drop radius. To demonstrate that field-driven separation can in fact occur, even in the presence of gravity, phase systems with very high droplet electrophoretic mobilities had to be developed. Suitable systems were found consisting of Sodium Dextran Sulfate (NaDS, $\overline{M}_w \sim 500{,}000$) and Pluronic® P-104 (a block co-polymer of poly (ethylene glycol) and poly (propylene glycol) in a weight ratio of 2:3, $\overline{M}_w = 5{,}400$) with potassium citrate as the supporting electrolyte. Two phase compositions were utilized: 8% w/w NaDS, 8% w/w P-104, 0.2M K_3 citrate (8/8/.2); and 5% w/w NaDS, 10% w/w P-104, 0.1M K_3 citrate (5/10/.1). Typical droplet mobilities for these systems were as shown in the table below for 6.5μ diameter drops.

	Mobility ($cm^2/v\text{-}5$)	
System	Bottom Phase Drops in Top Phase	Top Phase Drops in Bottom Phase
5/10/.1	$+8.8 \times 10^{-4}$	-8.4×10^{-4}
8/8/.2	$+17.6 \times 10^{-4}$	-15.3×10^{-4}

In these systems the top phase is rich in P-104 and the bottom is predominantly NaDS. To drive phase separation in coincidence with gravity, then, the electric field is applied with the anode at the top and the cathode at the bottom of the sample chamber.

A prototype phase separation chamber was constructed similar to that described in Section 4.2.1 of our original proposal. A cross-sectional view of the chamber is shown in Figure 1. The stirring screen was omitted, since mixed phase systems could be introduced directly into the chamber.

The apparatus consists of two electrode chambers containing bright Pt electrodes separated from a phase chamber (5cm L x 0.5 cm W x 0.2 cm deep) by two amicon XM-100 membranes. The phase chamber is split in half horizontally so that the two halves can be displaced laterally relative to one another, allowing isolation of the contents of each half. Feeder ports give access to the chamber for

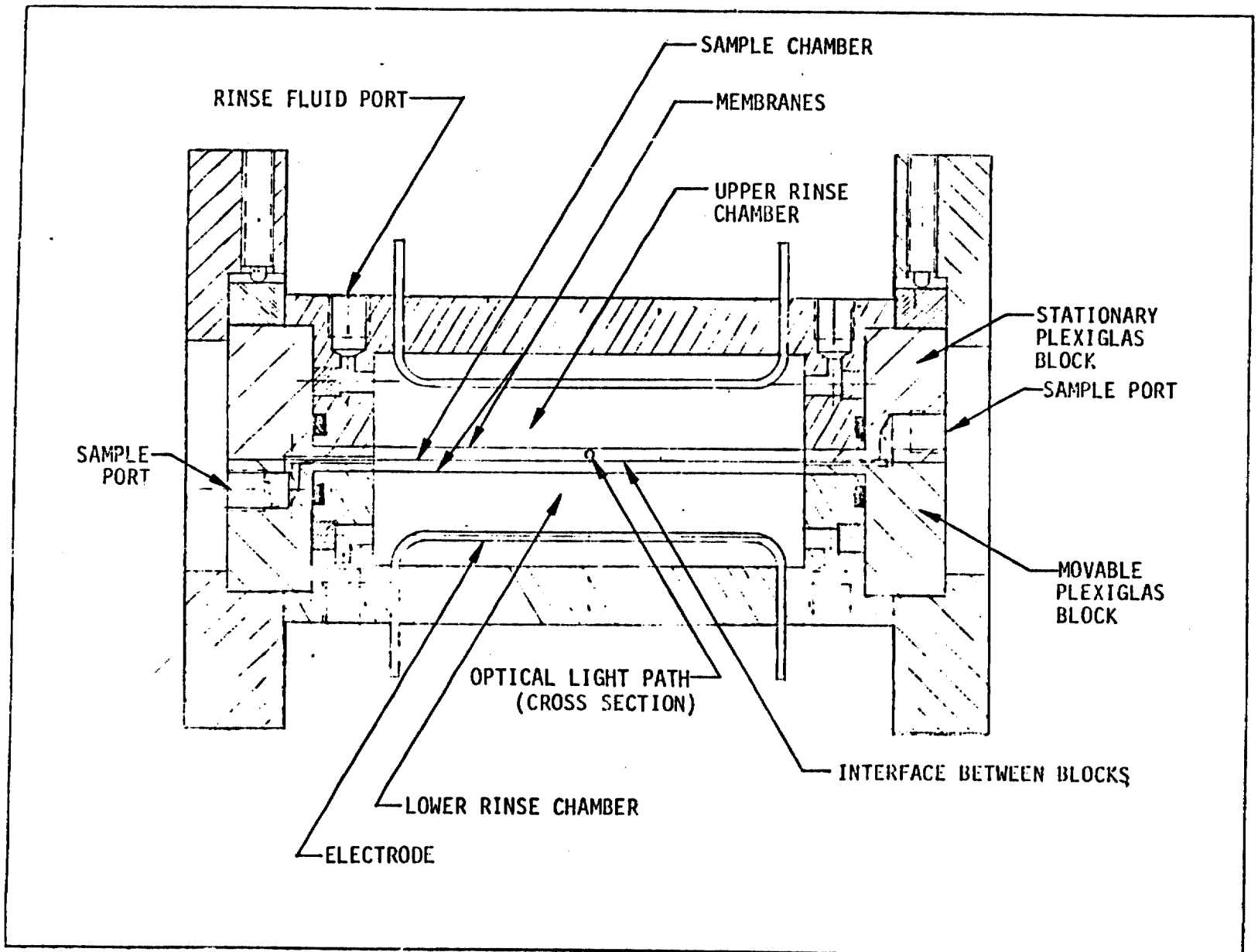

Figure 1. Prototype Phase Separation Chamber

filling and drainage in either the separated or contiguous configuration. Electrode rinse buffer is circulated through the upper and lower electrode chambers to remove electrode reaction products. The chamber and electrode assembly is made of poly (methyl methacrylate) lapped and polished to provide a good sliding seal between the upper and lower halves. The optical system used to follow phase separation turbidimetrically consists of a small ruby laser whose beam diameter is limited to ~0.03 cm by an entrance aperture. The beam traverses the width of the phase chamber at a vertical position which can be adjusted relative to the midline split. The beam intensity is measured with a solid state detector and amplifier after traversing the chamber and an 0.03 cm diameter exit aperture. The beam and apertures are aligned with water in the phase chamber so that when a turbid mixed phase system is present only non-scattered light is detected.

A typical trace of detector voltage as a function of time with no field applied is shown in Figure 2 for the 8/8/.2 system made up on a phase volume ratio of 9 parts top phase:1 part bottom phase. The beam samples all of the bottom phase and a portion of the top phase near the interface. At this phase volume ratio, the 1 g settling time is prolonged and the kinetics are easy to follow. The signal initially increases linearly in the early stages of phase coalescence, then fluctuates more severely as larger droplets form and the system separates supralinearly with time. At this amplifier gain setting for this run, full scale deflection of 100 mV corresponds to an essentially separated system. The degree of separation sufficient for all partition work corresponds to something less than this level (~50 mV) since residual drops smaller than cell dimensions have little effect on cell partition behavior yet still scatter a considerable amount of light.

The effect of applying an electric field of 5.1 v/cm to this system is shown in Figure 3. An immediate increase in the rate of separation is seen. The slope increases by a factor of about 35 and the intensity increases to an output of 20 mV (an arbitrary but convenient measure of separation time) in 1.1 min. as opposed to 10.5 min. in Figure 2.

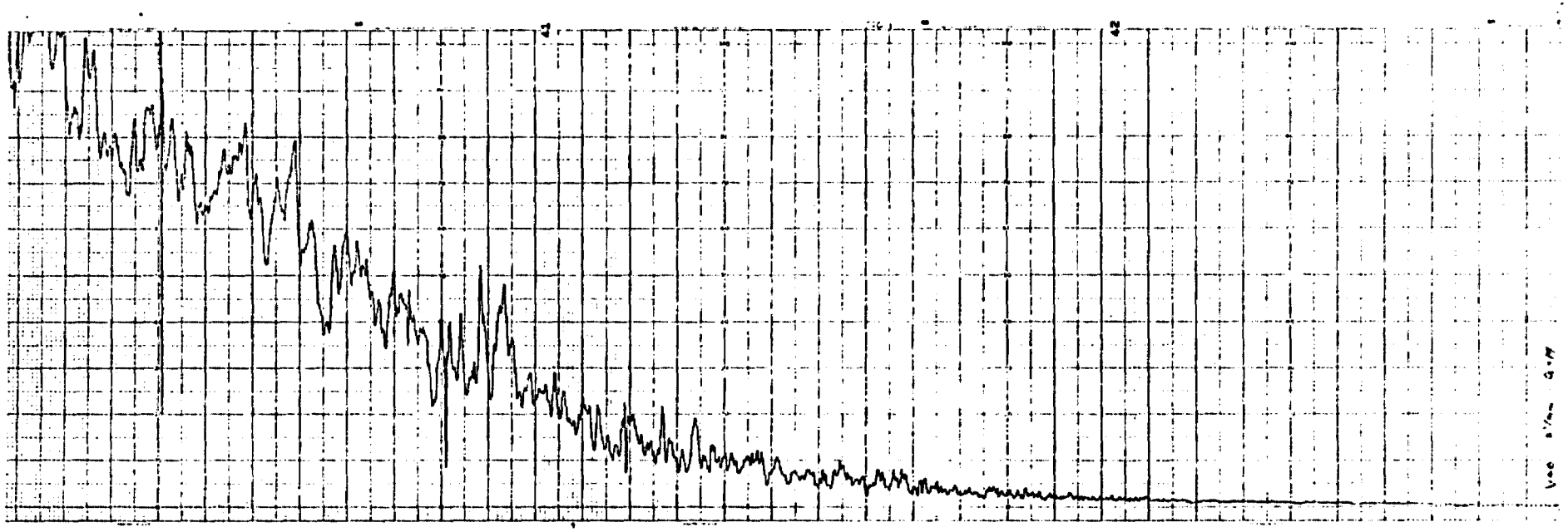

Figure 2. System 8/8/.2, Optical Detector Voltage Vs. Time, Chart Speed 2 "/min, Full- Scale Deflection = 100 mV. No Field Applied.

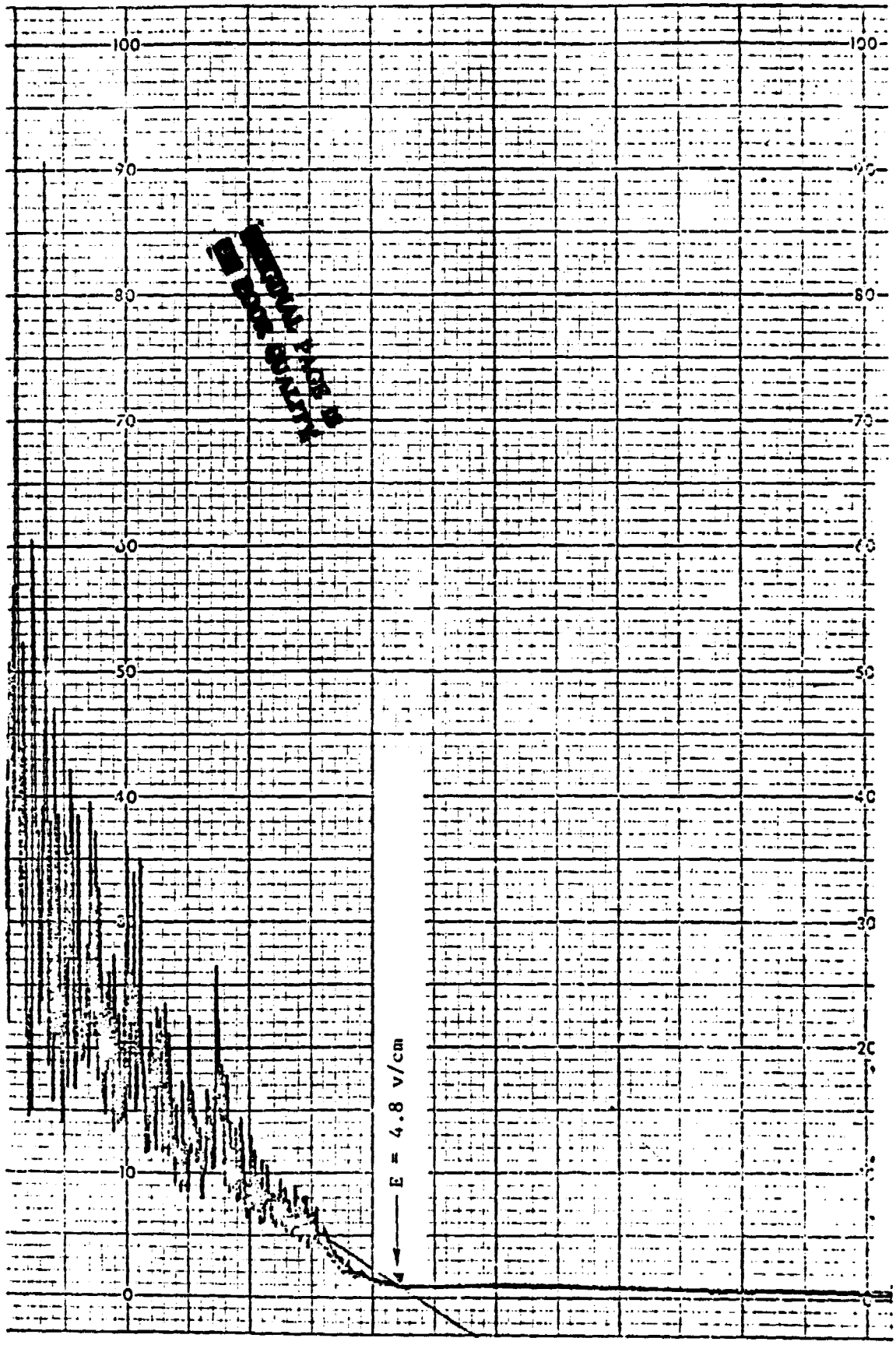

Figure 3. System 8/8/.2. Chart Speed and mV Scale as in Figure 2, Showing Field Transition from E=0 to E=4.8 v/cm.

Figure 4 gives another example of the field effect at ten times higher gain. As the field is increased from 0 to 2.8 v/cm, the separation rate increases until the field is turned off, at which point the rate slows. Re-application of 2.4 v/cm, then 3.4 v/cm rapidly clears the system.

Effects such as these were repeatedly seen in both the 8/8/.2 and 5/10/.1 systems, although the former always exhibited larger field effects (initial slopes 3 to 30 times greater) than the system with the lower droplet mobility. The effects could not have been due to unstable convection in the chamber since the Rayleigh number for these experiments was between 1 and 10 and the maximum temperature rise in the sample was less than 3°C. Moreover, the effect depended on the direction of the electric field; reversing its polarity induced mixing in a partially separated system, reducing the signal to zero. These results, then, are consistent with the presence of field-driven phase separation in these systems and represent, we believe, the first demonstration of this effect.

FIELD EFFECTS ON CELL SEPARATIONS

Since biological cells are known to exhibit characteristic electrophoretic mobilities, the possibility should be considered that an electric field applied to drive the phase separation could affect the cell partitioning process. Cell electrophoresis might, for instance, pull cells out of the interface and thus change their position in the phase system. We have as yet been unable to test this possibility directly since the NaDS/P-104/K_3 citrate system is highly non-physiological and damaging to cells. However, it seems likely that such effects will be absent or insignificant for the following reason: All biological cells under physiological conditions bear a negative surface charge and hence, will migrate upward in our apparatus.

In virtually all phase systems used in cell separation work, partition occurs between the top phase and the interface with no cells remaining in the bottom phase. With the electric field applied, then, cells which have partitioned into the top phase will only tend to move closer to the top electrode. Cells adsorbed in the interface will also tend to move in this direction, but the interfacial tension is sufficient to hold the cells in place. We have examined this point

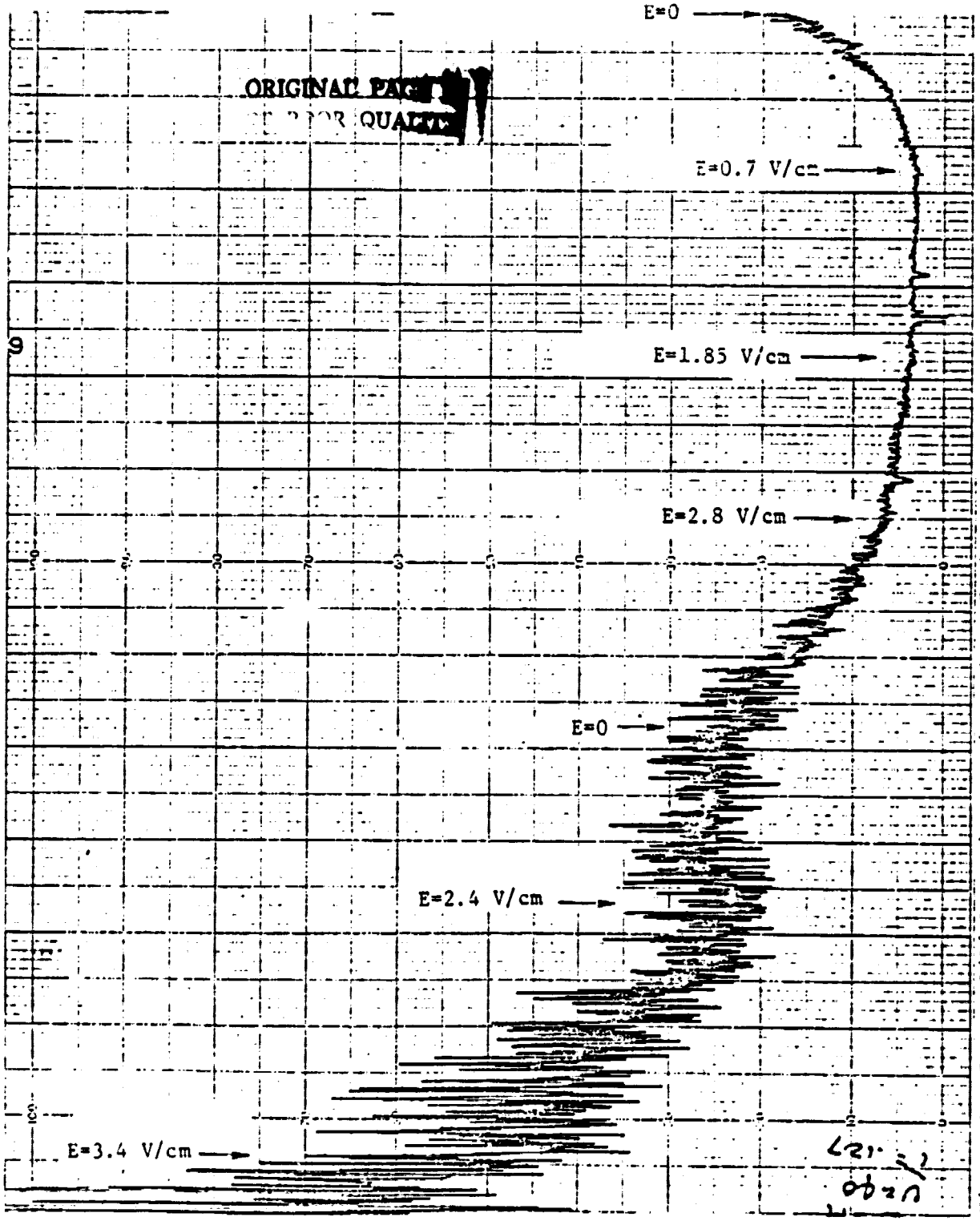

Figure 4. System 8/8/.2. Gain Ten Times that of Figures 2 and 3. Chart speed 2 in/min, showing effects of field strength changes as indicated.

by observing the electrophoresis of phase drops to which cells are adsorbed. At field strengths equal to those used here, the cells remained in the interface as the droplets moved at velocities one to two orders of magnitude greater than those at which the cells along would move in free suspension.. The latter velocities are very small compared to droplet velocities because of the relatively high phase viscosities and low cell surface potential. The interfacial tension (which is under experimental control) is apparently strong enough to maintain the relative position of the cell and interface under conditions similar to those present during field-driven phase spearation.

FIELD-DRIVEN PHASE SEPARATION TIME

At the phase volume ratios used in the present experiments, the NaDS/P-104/ citrate system separated in 1 to 2 min. at field strengths of 5 v/cm or less. Although we did not investigate the dependence of separation rate on phase volume ratio specifically, it was clear that systems having more nearly a 1:1 top:bottom ratio separated considerably faster, as would be expected. We could not operate at a 1:1 ratio with the NaDS/P-104 system because of a field-dependent instability associated with high contents of NaDS-rich bottom phase, which caused mixing. Presumably the instability is caused by the opposite mobilities of droplets and molecules of NaDS, a negatively charged polyelectrolyte. Molecules of NaDS in the separated bottom phase will move toward the anode while bottom phase droplets, which have a positive surface potential, migrate cathodically. No mixing was observed at smaller bottom-phase volume fractions, however. It is not anticipated that the observed instability will occur when both polymers are neutral which will be the case in the cell partition systems to be used in the flight experiments. Even if similar mixing is observed, it will still be possible to eliminate it by varying the phase volume ratio since only one bulk phase and the interface are actually occupied by cells after partition occurs.

Since we could not operate the demonstration system with equal top and bottom phase volumes, it was not possible to correlate directly the droplet mobilities with separation rates for systems corresponding to those to be used in the flight experiments. We can,. therefore, not directly calculate the separation times to be expected in a zero g experiment. However, an estimate may be made as follows:

When the phases separate after shaking, the interface forms fairly rapidly as the majority of the droplets coalesce into bulk phases. A haze of residual droplets is left in each phase which then settles and completes the separation. If these residual droplets are large enough (greater than cell dimensions), they can contain or adsorb cells and contribute to the final cell distribution. If they are about the size of or smaller than the cells being partitioned, they will not contribute and need not be allowed to settle before the cell distribution is determined or a transfer made in a countercurrent distribution experiment. An estimate of the effective separation time may then be obtained from the time required for droplets somewhat larger than cells to migrate through half the depth of the chamber. For gravitational settling, the settling time, t_{sep}, will therefore be given approximately by:

$$t_{sep} = \frac{d}{2v_{sed}} = \frac{d}{4 \cdot \Delta\rho \cdot g \cdot r^2} \left[\frac{2\eta_m + 3\eta_d}{\eta_m + \eta_d}\right]$$

where d = depth of chamber = 0.2 cm

$\Delta\rho$ = density difference between phases = $4 \times 10^{-2} g/cm^3$

g = 980 cm/s^2

r = drop radius

η_m = medium viscosity - 4×10^{-2}p

η_d = droplet viscosity = 0.3p

For r = 10μ (about 2x cell radius) this expression gives t_{sep} = 7.5 min., which corresponds closely to the observed time of about 5 min. in the CCD apparatus.

For field-driven separation, the appropriate velocity to use in (1) is the electrophoretic velocity $v = uE$, where u is the droplet mobility and E the applied electric field. Phase systems with high mobilities useful in cell partition work are currently under development but a conservative estimate for the mobility of such systems is $u = 0.6\ rs^{-1}$. The corresponding separation time in a 5 v/cm field for r = 10μ is $t_{sep} = d/2uE$ = 33 s. Accordingly, phase separation should occur very rapidly in the flight experiment. The separation period should therefore be readily compatible with the duration of reduced gravity available on a SPAR flight.

EQUIPMENT PERFORMANCE

It is clear from the results presented above that the apparatus performs satisfactorily. With a phase system in the sample chamber and an electrode rinse flow of 400 to 600 ml/min., a current of 0.5A±5% (corresponding to an electric field of about 13 v/cm in the sample chamber) can be maintained continuously for at least 15 minutes. There is occasional difficulty associated with foam in the electrode rinse after the buffer has re-cycled several times. The P-104 is quite surface active, and the small amount that leaches through the membranes can form stable foams during electrolysis that are somewhat difficult to remove. This problem should not occur in flight experiments, however, where poly (ethylene glycol), which has little surface activity, will replace the P-104.

The optical system employed also behaves satisfactorily. The read-out of detector voltage versus time provides an excellent record of the progression of phase separation, and it should prove feasible to follow the kinetics of separation even in the presence of high concentrations of cells.

CONCLUSION

We believe that the results cited above provide excellent evidence that the approach described in Beckman Proposal CS76-328, "Countercurrent Distribution of Biologicals", is valid. It would appear appropriate as a next step to test the cell separation under micro-gravity conditions. The experimental protocol we have outlined fits the requirements of a SPAR flight experiment particularly well. We feel that such an experiment is justified by the potential importance of this cell separation technique and the highly promising results now achieved in our preliminary experiments.